Soybean (Glycine max) allergy in Europe: Gly m 5 (beta-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy

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Abstract: BACKGROUND: Soybean is considered an important allergenic food, but published data on soybean allergens are controversial. OBJECTIVE: We sought to identify relevant soybean allergens and correlate the IgE-binding pattern to clinical characteristics in European patients with confirmed soy allergy. METHODS: IgE-reactive proteins were identified from a soybean cDNA expression library, purified from natural soybean source, or expressed in Escherichia coli. The IgE reactivity in 30 sera from subjects with a positive double-blind, placebo-controlled soybean challenge (n = 25) or a convincing history of anaphylaxis to soy (n = 5) was analyzed by ELISA or CAP-FEIA. RESULTS: All subunits of Gly m 5 (beta-conglycinin) and Gly m 6 (glycinin) were IgE-reactive: 53% (16/30) of the study subjects had specific IgE to at least 1 major storage protein, 43% (13/30) to Gly m 5 , and 36% (11/30) to Gly m 6. Gly m 5 was IgE-reactive in 5 of 5 and Gly m 6 in 3 of 5 children. IgE-binding to Gly m 5 or Gly m 6 was found in 86% (6/7) subjects with anaphylaxis to soy and in 55% (6/11) of subjects with moderate but only 33% (4/12) of subjects with mild soy-related symptoms. The odds ratio ($P < .05$) for severe versus mild allergic reactions in subjects with specific IgE to Gly m 5 or Gly m6 was 12/1. CONCLUSION: Sensitization to the soybean allergens Gly m 5 or Gly m 6 is potentially indicative for severe allergic reactions to soy.

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Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy

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Background

Soybean is considered an important allergenic food, but published data on soybean allergens are controversial.

Objective

We sought to identify relevant soybean allergens and correlate the IgE-binding pattern to clinical characteristics in European patients with confirmed soy allergy.

Methods

IgE-reactive proteins were identified from a soybean cDNA expression library, purified from natural soybean source, or expressed in *Escherichia coli*. The IgE reactivity in 30 sera from subjects with a positive double-blind, placebo-controlled soybean challenge (n = 25) or a convincing history of anaphylaxis to soy (n = 5) was analyzed by ELISA or CAP-FEIA.

Results

All subunits of Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) were IgE-reactive: 53% (16/30) of the study subjects had specific IgE to at least 1 major storage protein, 43% (13/30) to Gly m 5 , and 36% (11/30) to Gly m 6. Gly m 5 was IgE-reactive in 5 of 5 and Gly m 6 in 3 of 5 children. IgE-binding to Gly m 5 or Gly m 6 was found in 86% (6/7) subjects with anaphylaxis to soy and in 55% (6/11) of subjects with moderate but only 33% (4/12) of subjects with mild soy-related symptoms. The odds ratio ($P < .05$) for severe versus mild allergic reactions in subjects with specific IgE to Gly m 5 or Gly m6 was 12/1.

Conclusion

Sensitization to the soybean allergens Gly m 5 or Gly m 6 is potentially indicative for severe allergic reactions to soy.

Key words: Soybean allergy; soybean allergens; β-conglycinin; glycinin; DBPCFC; anaphylaxis; cDNA expression library

Abbreviations: AP, Alkaline phosphatase; BC, β-Conglycinin, Gly m 5; DBPCFC, Double-blind placebo-controlled food challenge; G, Glycinin, Gly m 6; IUIS, International Union of Immunological Societies; OAS, Oral allergy syndrome; OR, Odds ratio; pfu, Plaque-forming unit

Article Outline

Methods
Patients
SDS-PAGE and IgE immunoblot analysis
Establishment of a soybean cDNA expression library
IgE screening of the soybean cDNA expression library
Purification of natural BC subunits
N-terminal sequencing and LC-MS/MS
Recombinant proglycinin subunits
ELISA for soybean-specific IgE
Statistical analysis
Results
Screening of the cDNA expression library revealed IgE reactivity to various subunits of major soybean storage proteins
A complete battery of highly purified natural β-conglycinin subunits and recombinant proglycinins from soybean
IgE-binding to the soybean major storage proteins found in the majority of subjects with soybean allergy
Gly m 5 and Gly m 6 as potential diagnostic markers for severe allergic reactions to soybean
Discussion
Acknowledgements
Methods
A complete battery of highly purified natural β-conglycinin subunits and recombinant proglycinins from soybean
Reference
Fig E1. Appendix
Fig E2. Appendix
Table E1. Appendix
References

Soybean (Glycine max)–induced allergic symptoms may range from skin, gastrointestinal, or respiratory reactions to anaphylaxis.[1], [2] and [3] Food allergy to soy has been described primarily in young children with atopic dermatitis,[4], [5] and [6] but published data on the prevalence of soybean allergy in childhood are controversial. Moreover, the prevalence of soybean allergy in adults is still unknown.[7] and [8] Recently, we described the clinical characteristics of soybean allergy in 25 European adults and 5 children, in whom soy allergy was confirmed by a positive DBPCFC or according to a convincing history of anaphylaxis to soy.[3] Cumulative threshold doses in soybean DBPCFC were at least 1 order of magnitude higher than observed in peanut allergy,[9] and [10] but no correlation was found between the level of threshold doses and the severity of symptoms. In addition, the pattern of IgE reactivity determined by immunoblotting was highly individual and apparently did not correlate

with the severity of symptoms.[3] To date, at least 16 IgE-binding soybean proteins have been described, of which several have been characterized in more detail: the soybean Kunitz trypsin inhibitor,[11], [12] and [13] the thiol-protease Gly m Bd 30k that has been suggested as a major soybean allergen,[14], [15] and [16] the α subunit of the major storage protein β-conglycinin (BC),[17] the acidic chain of the major storage protein glycinin (G) G1 subunit,[18] and the basic chain of the G2 subunit.[19] Apart from a few exceptions, such as the work of Helm et al,[15], [16] and [19] most of the studies were based on subjects with atopic dermatitis whose food-related symptoms were not confirmed by DBPCFC. Moreover, IgE reactivity of the A5B3 glycinin was shown in children with cow's milk allergy but without confirmed soybean allergy.[20] Finally, the putative soybean allergen 2S albumin[13] showed no IgE-binding in 23 European subjects with soybean allergy.[21] Interestingly, only the soybean hull proteins Gly m 1 and Gly m 2 that are known as inhalant allergens in occupational or environmental soybean allergy as well as the birch pollen–related allergens Gly m 3 and Gly m 4 have been accepted officially as soybean allergens by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (http://www.allergen.org/Allergen.aspx). Unlike the legume peanut with at least 8 identified and officially accepted allergens, there is still a lack of knowledge on soybean allergens. The identification of relevant soybean allergens therefore remains an important task to achieve better understanding of the molecular mechanisms of soybean allergy with special regard to diagnosis, risk management, and future therapy. As an important step into this direction, we performed the identification of 2 soybean allergens, Gly m 5 (β-conglycinin) and Gly m 6 (glycinin), using serum samples of 30 subjects with soybean allergy.[3] The 2 soybean major storage proteins, β-conglycinin and glycinin, are 7 S and 11 S globulins and account for about 30% and 40% of the total seed proteins, respectively. In the vacuole, the mature form of β-conglycinin is accumulated into the densely packed state as trimers that are composed of 3 subunits, α (~67 kd), α′ (~71 kd), and β (~50 kd).[22] Glycinin is synthesized in the seeds during embryogenesis. It is a hexameric protein that is assembled by 5 different subunits, G1 (A1aB1b, 53.6 kd), G2 (A2B1a, 52.4 kd), G3 (A1bB2, 52.2 kd), G4 (A5A4B3, 61.2 kd), and G5 (A3B4, 55.4 kd). Each features at least 1 acidic (A) and basic (B) peptide chain that are linked by a disulfide bond.[23] Finally, the IgE reactivities to this complete panel of 8 subunits of the major storage

proteins were correlated to the clinical characteristics of our patient group to investigate the potential use as biomarkers for severe allergic reactions to soybean.

Methods

Patients

The patients were recruited in 3 European allergy centers—Zurich (Switzerland), Odense (Denmark), and Milan (Italy)—according to protocols approved by the Ethical Committees of the respective centers. The primary inclusion criterion was a positive food challenge (DBPCFC) with soy or a convincing history of anaphylactic reactions to soy. The clinical details of the study subjects have been published recently.[3] Challenges were discontinued after the dose leading to objective allergic symptoms or ingestion of the whole meal of 50 g soy (≙26.5 mg soy protein). The lowest observed adverse effect levels for subjective and for objective allergic reactions were 5.3 mg and 240.6 mg of soy protein, respectively. The most relevant data necessary for interpretation of the *in vitro* results obtained in this study are summarized in Table I. To set uniform and comparable criteria for the depicted most severe symptom, symptoms were recorded as experienced under challenge. In addition, a convincing history of anaphylaxis, evaluated by a standardized case report form, was accepted. The severity of symptoms on soy ingestion was graded as follows: (1) mild: symptoms such as oral allergy syndrome (OAS), tightness of the throat, nausea, gastrointestinal pain, or dyspnea without monitored drop in FEV_1; (2) moderate: symptoms such as rhinitis, flush, urticaria, angioedema, diarrhea, or emesis; and (3) severe: drop of blood pressure, or life-threatening laryngeal edema.

Table I.

Clinical characteristics of the study subjects with soybean allergy

		Soybean allergy					CAP (kU/L)		
Patient no.	**Age (y)/sex***	**Clinical evidence**	**Most severe symptom challenge/history‡**	**Graded symptoms**		**Peanut allergy according to history**	**Soy extract**	**Soy Glym 4**	**Peanut extract**
1	31/M	History	Drop of blood	Sever	Objecti	Yes	2.1	<0.	2.3

		Soybean allergy					CAP (kU/L)		
Patient no.	**Age (y)/sex***	**Clinical evidence**	**Most severe symptom challenge/history†**	**Graded symptoms**		**Peanut allergy according to history**	**Soy extract**	**Soy Glym 4**	**Peanut extract**
		of anaphylaxis to soy	pressure	e	ve			35	
2	27/M	Soybean DBPCFC	Rhinitis	Moderate	Objective	Yes	<0.35	3.2	<0.35
3	32/F	Soybean DBPCFC	Drop of blood pressure	Severe	Objective	Yes	0.5	4.0	0.8
4	3/F	Soybean DBPCFC	Angioedema, flush	Moderate	Objective	No	<0.35	3.1	<0.35
5	18/F	Soybean DBPCFC	Urticaria	Moderate	Objective	Yes	0.4	4.0	1.6
6	22/F	Soybean DBPCFC†	Angioedema	Moderate	Objective	Yes	6.4	<0.35	>100
7	17/F	Soybean DBPCFC	OAS, tightness of throat	Mild	Subjective	Yes	<0.35	7.2	0.5
8	9/M	History of anaphylaxis to soy	Laryngeal edema	Severe	Objective	Yes	15.9	<0.35	2.3
9	40/F	Soybean DBPCFC	Flush	Moderate	Objective	No	<0.35	1.0	<0.35
10	32/F	Soybea	Flush	Moder	Objecti	Yes	1.6	9.3	4.7

		Soybean allergy					CAP (kU/L)		
Patient no.	**Age (y)/sex***	**Clinical evidence**	**Most severe symptom challenge/history†**	**Graded symptoms**		**Peanut allergy according to history**	**Soy extract**	**Soy Glym 4**	**Peanut extract**
		n DBPCFC		ate	ve				
11	21/M	Soybean DBPCFC‡	OAS, dysphagia	Mild	Subjective	Yes	0.5	10.4	1.1
12	23/F	Soybean DBPCFC	OAS, >20% decrease in FEV_1	Moderate	Objective	Yes	0.4	1.8	91.9
13	39/F	Soybean DBPCFC	OAS, nausea	Mild	Subjective	No	<0.35	0.4	<0.35
14	35/M	Soybean DBPCFC	OAS, nausea	Mild	Subjective	Yes	1.5	6.1	62.9
15	22/F	Soybean DBPCFC	OAS, nausea	Mild	Subjective	Yes	1.3	15.4	3.2
16	32/M	Soybean DBPCFC	OAS	Mild	Subjective	Yes	2.1	77.7	6.5
17	18/M	Soybean DBPCFC	OAS, nausea	Mild	Subjective	No	1.8	22.8	5.3
18	14/M	Soybean DBPCFC	Diarrhea	Moderate	Objective	Yes	10.0	4.1	21.1
19	11/F	Soybean DBPCF	OAS	Mild	Subjective	No	20.8	11.2	19.0

		Soybean allergy					CAP (kU/L)		
Patient no.	**Age (y)/sex***	**Clinical evidence**	**Most severe symptom challenge/history†**	**Graded symptoms**		**Peanut allergy according to history**	**Soy extract**	**Soy Glym 4**	**Peanut extract**
		C							
20	6/M	Soybean open challenge	Emesis	Moderate	Objective	No	4.7	<0.35	1.8
21	9/F	Soybean DBPCFC	OAS, GIT	Mild	Subjective	Yes	18.1	>100	>100
22	44/F	History of anaphylaxis to soy	Drop of blood pressure	Severe	Objective	No	<0.35	17.2	4.4
23	16/F	Soybean DBPCFC	Flush, urticaria	Moderate	Objective	Yes	2.5	<0.35	7.4
24	28/M	Soybean DBPCFC	OAS	Mild	Subjective	Yes	3.1	<0.35	<0.35
25	69/F	Soybean DBPCFC	Flush	Moderate	Objective	No	<0.35	<0.35	<0.35
26	44/F	Soybean DBPCFC	GIT	Mild	Subjective	Yes	0.7	6.3	5.8
27	30/F	History of anaphylaxis to soy	Drop of blood pressure	Severe	Objective	Yes	2.9	<0.35	8.6
28	24/M	History of anaphyl	Drop of blood pressure	Severe	Objective	Yes	52.5	0.6	>100

		Soybean allergy					CAP (kU/L)		
Patient no.	**Age (y)/sex***	**Clinical evidence**	**Most severe symptom challenge/history‡**	**Graded symptoms**		**Peanut allergy according to history**	**Soy extract**	**Soy Glym 4**	**Peanut extract**
		axis to soy							
29	63/F	Soybean DBPCFC	GIT	Mild	Subjective	No	1.2	2.4	1.5
30	39/M	Soybean DBPCFC	Drop of blood pressure	Severe	Objective	No	64.9	<0.35	20.6

Full-size table

Patients 1-18 and 28, Switzerland; patients 19-21, Denmark; patients 22-27, 29, and 30, Italy.

F, Female; *GIT,* gastrointestinal; *M,* male.

* Age at time of blood drawing and challenge.

† Challenge vehicle: chocolate drink instead of chocolate bar.

‡ Most severe symptom in challenge or according to anaphylaxis history.

SDS-PAGE and IgE immunoblot analysis

SDS-PAGE was performed under reducing conditions, and IgE immunoblot analysis was performed as recently described.[3] Soybean extract was subjected to SDS-PAGE at 20 μg/cm gel, purified natural BC was analyzed at 7.5 μg/cm with 2.5 μg/cm of each subunit (α, α′, and β), and recombinant proglycinin was analyzed at 10 μg/cm with each subunit (A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3) at 2 μg/cm. IgE reactivities were detected with horseradish peroxidase on polyclonal anti-IgE and visualized by chemiluminescence on x-ray–sensitive film. Alternatively, alkaline phosphatase (AP)–conjugated monoclonal anti-IgE (Pharmingen, San Diego, Calif; dilution 1:1000, 4 hours) and colorimetric staining with an AP-substrate solution (Bio-

Rad Laboratories, Hercules, Calif) were applied. Silver staining of gels was performed according to Heukeshoven and Dernick.[24]

Establishment of a soybean cDNA expression library

Total RNA was isolated from freeze-dried almost ripened but slightly green soybean seeds as previously described by Hoff et al.[25] Five micrograms of poly(A) mRNA were purified from the isolated total RNA fraction by the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany). After reverse transcription and addition of *Eco*R I/*Hind* III linker arms, the cDNA was size-fractionated (Mini Column Fractionation Kit, Novagen, Madison, Wis) with fragments of as many as 3000 bases and a major portion of 1000 to 2000 bases in length. A cDNA expression library was established by using the λSCREEN-1 system (Novagen) according to the manufacturer's instructions (technical bulletin TB119 02/99). The most prominent cDNA fraction of approximately 300 to 2000 bases in length was directionally ligated into λSCREEN vector and *in vitro* packed. The primary library had a titer of 4.5×10^5 plaque-forming units per milliliter (pfu/mL; total library size, 2.25×10^5 pfu) and was amplified to 4×10^9 pfu/mL.

IgE screening of the soybean cDNA expression library

For immunoscreening, 2×10^4 pfu was plated on 90-mm plates, incubated at 37°C until plaques appeared (5-7 hours), and overlaid with nitrocellulose filters (Hybond C; GE Healthcare, Munich, Germany; 4°C; overnight). The nitrocellulose plate lifts were blocked in 50 mmol/L TRIS-buffered saline (pH 7.4) containing 1% gelatin and 0.1% Tween 20 and incubated with patient serum no. 28 (diluted 1:10, overnight). The bound IgE was visualized with anti–IgE-AP as described. Nonspecific binding was reduced by serum preincubation with equal volumes of suspension and lysate of BL21(DE3)pLysE-plating cells. IgE-reactive phage recombinants were screened by PCR with SP6 promoter and T7 terminator primers. The purified PCR products were sequenced.

Purification of natural BC subunits

Soybean extract was prepared in 10 mmol/L PBS (pH 7.4) as recently described.[3] Natural α, α′, and β subunits of BC were purified from soybean extract by preparative

SDS-PAGE (model 491 Prep-Cell; Bio-Rad Laboratories, Munich, Germany) as described by Reese et al.[26] As much as 50 mg total protein from soybean extract was separated in the 28-mm internal diameter column with a 25-mm-high stacking gel (5% total acrylamide concentration; 1.5% cross-linker acrylamide concentration) and a 65-mm-high separation gel (7,5% T, 1.5% C). Fractions containing the purified single subunits were identified by IgE Western blotting, pooled, and dialyzed against 3-(N-morpholino)-propanesulfonic acid buffer (20 mmol/L 3-(N-morpholino)-propanesulfonic acid, 8 mmol/L sodium acetate, 1 mmol/L 2-(bis [carboxymethyl] amino) acetic acid, pH 7.0). The purity of the isolated BC subunits was checked by SDS-PAGE and silver staining, and purity and identity were verified by N-terminal sequencing and liquid chromatography tandem mass spectrometry.

N-terminal sequencing and LC-MS/MS

N-terminal sequencing analyses were performed on a Procise 492 protein sequencer (Applied Biosystems, Monza, Italy) after protein adsorption on ProSorb PVDF membranes (Applied Biosystems). For LC-MS/MS analyses, the spots cut from the gel were destained and the proteins trypsinized as described by Hellmann et al.[27] The tryptic mixtures were analyzed by LC-MS/MS as previously described.[28]

Recombinant proglycinin subunits

The recombinant proglycinin subunits G1 (A1aB1b, 53.6 kd), G2 (A2B1a, 52.4 kd), G3 (A1bB2, 52.2 kd), G4 (A5A4B3, 61.2 kd), and G5 (A3B4, 55.4 kd) were generated and purified as summarized elsewhere in detail.[23]

ELISA for soybean-specific IgE

Microtiter plates (Maxisorp F96, Nunc, Thermo Fisher Scientific, Langenselbold, Germany) were coated with either a mixture of 50 ng of each BC subunit or a mixture of 50 ng of each proglycinin subunit in 50 mmol/L carbonate buffer (pH 9.6) per well overnight. Human myeloma IgE (Biogenesis via AbD Serotec, Duesseldorf, Germany) was coated simultaneously to prepare a standard curve between 0.1 and 218.7 ng IgE in a 3-fold dilution series. Excess binding sites were blocked with 2% BSA in 10 mmol/L PBS for 2 hours. Human sera, diluted at 1:4 in incubation buffer (10 mmol/L PBS containing 2% BSA and 0.05% Tween 20, pH 7.4) were added to

wells coated with allergens (2 hours). Standard wells were incubated with incubation buffer only. Immunodetection was performed with biotin-conjugated antihuman IgE from goat (lot WJ 138; KPL Inc., Gaithersburg, Md) and horseradish peroxidase–conjugated NeutrAvidin (Pierce, Rockford, Ill) each for 60 minutes (1:2000 and 1:10,000, respectively). Enzymatic staining was performed with tetramethylbenzidine as described elsewhere.[29] Plates were read at 450 nm (630 nm reference) wavelength and subtracted by the OD of the buffer blank. A semi-log sigmoidal IgE standard curve was fitted by the SOFTmax PRO software (version 2.4.1; Molecular Devices, Sunnyvale, Calif). Maximum ODs were calculated from asymptotal extrapolation to infinite concentration. The ODs of the soy allergic sera (0.05-2.8) as well as of a nonallergic serum pool (n = 3; OD 0.05) were calculated as percentage of the maximum OD (3.2) of the myeloma IgE to achieve comparability of the relative IgE-binding capacities between BC and proglycinin. The results were interpreted only in a qualitative manner: the IgE-binding to the soybean major storage proteins was considered positive if equal or greater than twice the reading of the nonallergic serum pool.

Statistical analysis

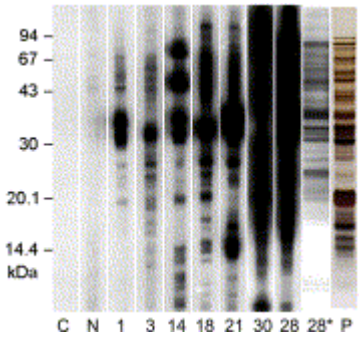
Statistical data analysis was performed using SAS/STAT software (SAS Institute Inc, Cary, NC). The odds ratios (ORs) were calculated within a 95% CI. Statistical significance was accepted at a probability value (Wald χ^2) of $P < .05$.

Results

Screening of the cDNA expression library revealed IgE reactivity to various subunits of major soybean storage proteins

For IgE immunoscreening of the soybean cDNA expression library, a representative soy-allergic serum had to be identified. A selection of sera showing an IgE-binding pattern that is representative for the soybean allergogram of the 30 study subjects[3] is shown in Fig 1. In particular, the sera of patients 28 and 30 had very high levels of soybean-specific IgE and displayed a prominent IgE-binding over a broad range of molecular weight that covered the majority of IgE-binding soybean proteins (Fig 1). For a better comparability of the IgE-binding pattern of serum 28 with others, the resolution of immunodetection was improved while the sensitivity was decreased

(lane 28*). Therewith, serum 28 was identified as a suitable candidate for immunoscreening. Furthermore, study subject 28 agreed on a separate blood donation to provide enough serum for IgE screening of the whole library.



Full-size image (23K)

Fig 1. IgE-immunoblot analysis of a soybean extract. All lanes except for 28* and P were detected with peroxidase mediated chemiluminescence (*lane C:* buffer control; *lane N:* nonatopic control serum pool [n = 3]; *lanes 1, 3, 14, 18, 21, 30, and 28:* sera from subjects with soybean allergy summarized in Table I; *lane 28*:* patient 28 detected with phosphatase and precipitating dye; *lane P:* silver-stained total soybean protein).

For a primary screening of the entire soybean expression library, 20 plates with 2 × 10^4 pfu/plate were immunodetected with serum 28. In total, 23 IgE-reactive primary clones were selected and underwent further rounds of enrichment and screening for IgE reactivity until pure clones of an IgE-reactive primary plaque were obtained. The cDNA sequences of positive clones were determined and translated amino acid sequences subjected to a Basic Local Alignment Search Tool search by using the algorithm of Altschul et al[30] (status February 2007). The majority of IgE-reactive clones referred to cDNA of the major storage protein BC (see this article's Table E1 in the Online Repository at www.jacionline.org). Expressed partial peptides of all 3 subunits (α, α′, and β) were IgE-reactive. Of the storage protein glycinin, expressed fragments of 2 (G1, G2) of the 5 major subunits were IgE-reactive. The sequenced cDNA inserts ranged from 304 to 736 bp and encoded for partial sequences with

approximately 15% (for the BC α′ subunit) and 54% (for the BC β subunit) of primary amino acid sequence coverage.

A complete battery of highly purified natural β-conglycinin subunits and recombinant proglycinins from soybean

All 3 subunits (α, α′, β) of the soybean major storage protein BC were purified from a natural soybean source. The proglycinins G1 to G5 were obtained by heterologous expression in *Escherichia coli*. All proteins featured high purity and convincing IgE-binding characteristics. Details are given in this article's Figs E1 and E2 in the Online Repository at www.jacionline.org.

IgE-binding to the soybean major storage proteins found in the majority of subjects with soybean allergy

The frequency of sensitization to the soybean major storage proteins BC and G in our study population was analyzed by IgE-ELISA. β-Conglycinin, composed of the 3 subunits α, α′, and β, and glycinin, composed of the proglycinins G1, G3, G4, and G5, were investigated in ELISA as either BC or G protein mixtures coupled to the solid phase. The G2 proglycinin was not included because of a low solubility in the coating buffer, resulting in precipitation of the protein. The results are given in Table II. The IgE ELISA proved to be adequately sensitive, because even in sera showing weak reactivity on immunoblots and in the CAP system, a clearly positive signal was obtained (eg, numbers 1, 2, 5, and 20).

Table II.

IgE reactivity of purified natural Gly m 5 and recombinant Gly m 6 in ELISA (N, nonatopic control serum pool)

	Gly m 5 (β-conglycinin)		**Gly m 6 (glycinin)**	
Patient no.	**% OD**	**IgE reactivity**	**% OD**	**IgE reactivity**
1	6.7	+	29.8	+
2	1.3		3.5	+

	Gly m 5 (β-conglycinin)		Gly m 6 (glycinin)	
Patient no.	**% OD**	**IgE reactivity**	**% OD**	**IgE reactivity**
3	2.7	+	2.2	
4	1.2		1.8	
5	4.1	+	2.4	
6	4.1	+	83.0	+
7	1.0		1.5	
8	63.6	+	3.1	+
9	0.8		2.0	
10	1.3		2.3	
11	0.6		1.9	
12	1.1		6.1	+
13	1.2		2.0	
14	24.7	+	17.0	+
15	3.1	+	1.7	
16	1.1		1.6	
17	1.3		2.2	
18	10.8	+	1.7	
19	11.0	+	2.8	+
20	2.4	+	1.6	
21	6.6	+	27.3	+
22	0.7		2.2	
23	1.1		1.8	
24	1.7		1.5	
25	0.5		2.0	
26	1.4		2.6	
27	0.9		3.2	+
28	69.8	+	87.8	+

	Gly m 5 (β-conglycinin)		Gly m 6 (glycinin)	
Patient no.	**% OD**	**IgE reactivity**	**% OD**	**IgE reactivity**
29	1.0		1.7	
30	77.7	+	61.1	+
N	1.0		1.4	
Cutoff	2.0		2.8	

±, Positive IgE binding equal to or greater than cutoff.

Forty-three percent (13/30) of subjects had specific IgE to BC and 37% (11/30) to G. All of the 5 children (age ≤14 years) participating in the study (numbers 8, 18, 19, 20, and 21) had specific IgE to BC and 3 (60%) to G. In total, 53% (16/30) of the subjects with soybean allergy had specific IgE to 1 or more soybean major storage proteins. Thus, the official nomenclature was applied to BC (Gly m 5) and G (Gly m 6) by the IUIS Allergen Nomenclature Subcommittee. The subunits of the oligomeric allergens were designated as Gly m 5.0101 (α), Gly m 5.0201 (α′), Gly m 5.0301/Gly m5.0302 (β), Gly m 6.0101 (G1), Gly m 6.0201 (G2), Gly m 6.0301 (G3), Gly m 6.0401 (G4), and Gly m 6.0501 (G5), following the rules for isoforms.

Gly m 5 and Gly m 6 as potential diagnostic markers for severe allergic reactions to soybean

According to the symptom classification, 12 (40%) subjects had mild allergic symptoms against soy, 11 (37%) had moderate reactions, and 7 (23%) had severe soy-related allergic reactions (Table I). Of those 7 subjects with severe soy-related allergic reactions, only 2 did not have a peanut allergy (according to history). Six of 7 patients (86%) with anaphylaxis either by history or during soybean challenge (numbers 1, 3, 8, 22, 27, 28, and 30) were sensitized to Gly m 5 or Gly m 6. By contrast, only 4 of 12 (33%) subjects who experienced mild subjective symptoms (numbers 7, 11, 13-17, 19, 21, 24, 26, and 29) had specific IgE to the soybean major storage proteins. However, 11 (92%) of these subjects had IgE specific to the birch pollen–related soy allergen Gly m 4.

Therefore, we calculated the ORs within a 95% CI in relation to the presence of specific serum IgE for the events “severe versus mild” and “severe versus moderate or mild” allergic reactions, respectively (Table III). Statistical significance with $P < .05$ was given for the following events: we calculated an OR of 7.08 for the event of having a severe rather than a moderate or mild allergic reaction in the presence of IgE specific to proglycinin. Moreover, the event of rather having a severe than a mild soy-related allergic reaction was given an OR of 12.00 if specific IgE to Gly m 5 or Gly m 6 was detectable. By contrast, we calculated an OR of 0.07 (1/14) for the event of having a severe rather than a mild soy-related allergic reaction in those subjects with detectable Gly m 4 specific IgE. Interestingly, the presence and concentration of soybean specific serum IgE determined by CAP-FEIA did not give a statistically significant increase of the OR for both events, “severe versus mild” and “severe versus moderate or mild.”

Table III.

ORs for the events of severe versus mild, and severe versus moderate or mild allergic reactions, depending on the presence of soy allergen specific IgE

Specific IgE	**Comparison**	**OR**	***P* value**
Gly m 5	Severe vs mild	5.00	.121
(ELISA)	Severe vs moderate/mild	4.69	.102
Gly m 6	Severe vs mild	7.50	.060
(ELISA)	Severe vs moderate/mild	7.08	.042
Gly m 5 or	Severe vs mild	12.00	.049
Gly m 6 (ELISA)	Severe vs moderate/mild	7.80	.076
Gly m 4	Severe vs mild	0.07	.038
(CAP)	Severe vs moderate/mild	0.21	.087
Soy extract	Severe vs mild	1.20	.891
(CAP)	Severe vs moderate/mild	2.12	.525

Discussion

Published data on soy allergens are still controversial, and no decision points of specific IgE for predicting clinical reactivity to soy were found.[3] and [4] To increase our knowledge on soy allergy at the molecular level and develop improved diagnostic tools for soy allergy, a directional cDNA expression library from developing soybean seeds was established. The library was screened with the serum of a subject with soybean allergy with a convincing history of anaphylaxis to soy and strong IgE reactivity to the majority of bands recognized by the other 29 sera of subjects with soy allergy.

For the glycinins, heterologous expression of the 5 proglycinins appeared to be more straightforward than the purification of 6 acidic and 5 basic subunits from natural soybean source. By contrast, the 3 subunits of BC were easily accessible by purification in comparison with heterologous expression. Testing sera from our subjects with soybean allergy demonstrated for the first time IgE-binding to all subunits of BC (Gly m 5) and G (Gly m 6) (Figs E1 and E2). To our knowledge, this *in vitro* study on soybean allergens enrolls the largest number of clinically confirmed subjects with soybean allergy (n = 30) who were tested for IgE reactivity to a large panel of purified soybean allergens. In total, 53% (16/30) of the subjects with soybean allergy had specific IgE to 1 or more soybean major storage proteins, including 13 (43%) with specific IgE to Gly m 5 and 11 (36%) with IgE to Gly m 6. Neither Gly m 5 nor Gly m 6 was a major allergen in our study population according to the IUIS criteria. Also, the overall sensitivity of soybean CAP-FEIA in our patients with soybean allergy was 77% (23/30) and clearly below the already published sensitivity in children with soy allergy.[4] Our study patients exhibited fairly low levels of soybean-specific IgE in CAP-FEIA, and of the 7 sera without specific IgE to soybean extract (< 0.35 kU/L), 6 showed IgE-binding to Gly m 4, the homolog to the birch pollen major allergen Bet v 1. These observations parallels the findings that in birch pollen–related soybean allergy mediated by Gly m 4, diagnosis is hampered by the fairly low levels of Gly m 4 in soybean extracts.[2]

The depicted most severe symptoms were recorded as experienced under challenge. Subjects with a history of soy anaphylaxis were not challenged for ethical reasons. Because we did not want to miss the most severe reactors, a convincing history of anaphylaxis was accepted if approved by all clinical centers. Other symptoms

according to case history were not considered to apply the most uniform criteria to evaluation of symptoms and subsequent correlation to allergen sensitization profiles.

Interestingly, a sensitization to the storage proteins Gly m 5 and Gly m 6 was found in 6 of 7 (86%) subjects with anaphylaxis either by history or occurrence during food challenge with soybean. Recently, the sensitizing and anaphylactic potential of the soybean major storage proteins was demonstrated in an animal model with BALB/c mice sensitized and challenged with purified natural Gly m 5 and Gly m 6.[31]

By contrast, only 4 of 12 (33%) study subjects who experienced mild subjective symptoms had IgE specific for the soybean major storage proteins. However, 11 (92%) of the subjects who experienced mild subjective symptoms had specific IgE to the birch pollen–related soy allergen Gly m 4.

In our group of subjects with soybean allergy, we did not find any correlation between the level of specific IgE to soybean in CAP-FEIA and the severity of symptoms of soybean allergy.[3] This finding is in full agreement with published data on soybean specific IgE levels in children and adolescents.[4] and [6] Moreover, the presence of specific serum IgE to soybean extract according to CAP-FEIA analysis did not give a statistically significant rise of the OR for the event of having a severe or moderate rather than a mild soy-related allergic reaction. However, by analyzing the IgE reactivity of our subjects with soybean allergy to soybean major storage proteins, a more distinct discrimination between mild and severe allergic reactions became evident. When calculating the ORs for the event of having a severe rather than a mild allergic reaction depending on the status of allergen sensitization, we found with statistical significance ($P < .05$) a high ratio (12/1) for those subjects with specific IgE to 1 or more of the soybean major storage proteins. By contrast, the OR for mild versus severe allergic reactions was 14 to 1 in subjects with sensitization to Gly m 4. Consequently, specific IgE to 1 or more soybean major storage proteins indicates a high risk of having a potentially severe allergic reaction to soy.

Moreover, in the 5 children with soy allergy, we found a high frequency (>50%) of IgE-binding to Gly m 6 and especially to Gly m 5. Thus, the soybean major storage proteins are putative major soybean allergens in children.

Two studies provided evidence that Gly m 4 is able to cause severe allergic reactions to soy in subjects with birch pollen allergy.[2] and [32] However, compared with the strong link to the soy storage proteins in the current study, this was not evident for Gly m 4. We believe that this may be explained by the fact that Kleine-Tebbe et al[32] investigated a population of subjects with birch pollen allergy preselected for severe reactions to soy. In the study by Mittag et al,[2] a lower frequency of systemic reactions was observed, but evidence was provided that approximately 10% of subjects with birch pollen allergy with specific IgE of at least 17.5 kU/L to Bet v 1 are clinically allergic to soybean. It may still be premature to conduct a relative risk assessment of Gly m 4 versus the soy storage proteins. On one hand, the current study clearly shows a higher risk of serious reactions in patients IgE-positive to Gly m 5 or Gly m 6 compared with Gly m 4, indicating a lower allergenicity per se of Gly m 4. On the other hand, this allergen can indeed induce severe reactions, in particular in patients with high IgE titers to Bet v 1, and on a population-based level, such patients may be far more numerous than Gly m 5±Gly m 6–positive subjects in central Europe. In this study about soybean allergens, soybean allergy of the underlying subjects was clinically confirmed in DBPCFC with soy-containing chocolate and chocolate drink, respectively. In these highly processed food matrixes, the heat-labile Gly m 4 may be denatured to a certain degree, thus facilitating less allergenic potential than the major soybean storage proteins. Finally, because case history was not included in grading symptom severity in challenged subjects, an underestimation of symptom severity cannot be fully excluded, in particular in those subjects with a predominant IgE reactivity to Gly m 4.

In summary, we conclude from the current study that a sensitization to the soybean major storage proteins Gly m 5 or Gly m 6 is likely to result in severe reactions to soybean, and therefore, component-resolved *in vitro* testing with these molecules in purified form can provide a diagnostic marker to identify subjects with soy allergy who are at high risk for severe clinical symptoms. Nonetheless, in areas with birch trees, primary sensitization to Bet v 1 with potential clinical cross-reactivity to soybean Gly m 4, especially in soy products with a low processing level, still needs to be taken into account when assessing the risk for severe soybean-related allergic reactions.

Clinical implications

Component-resolved diagnosis with purified Gly m 5 and Gly m 6 is potentially applicable for identifying subjects at risk of experiencing severe soy-related allergic reactions.

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Methods

A complete battery of highly purified natural β-conglycinin subunits and recombinant proglycinins from soybean

All 3 subunits (β, α, α′) of the major storage protein BC were purified from soybean extract by preparative SDS-PAGE, and the purity was verified by analytical SDS-PAGE and silver staining (Fig E1, lanes β, α, and α′), LC-MS/MS, and N-terminal sequencing. The N-terminal sequences were identical to the mature subunits α (VEKEE), α′ (VEEEE), and β (LKVRE). The identity of natural β-conglycinin was further confirmed by nano-electrospray ionization MS/MS analysis followed by a MS/MS database search using Mascot. The amino acid sequence coverage obtained for the α, α′, and β subunits of natural purified BC was 35%, 37%, and 48%, respectively. The α (Gly m 5.0101) and α′ (Gly m 5.0201) subunits were identified referring to the database entries gi|9967357 and gi|9967361, respectively. The β subunit (Gly m 5.03) displayed 2 variants with 99.5% identity, refering to the database entries gi|21465630 (Gly m 5.0301) and gi|21465633 (Gly m 5.0302).

All 3 subunits (Gly m 5.01, Gly m 5.02, and Gly m 5.03) were IgE-reactive in the immunoblot analysis of a mixture of the purified Gly m 5 subunits with selected patients' sera (Fig E1). Each of the subunits bound IgE from serum 28 when investigated as single protein (data not shown). Despite a broad range of IgE-reactive proteins in soybean extract (Fig 1) no other IgE-binding soybean proteins were present in the purified preparations of Gly m 5 subunits (Fig E1).

The recombinant proglycinin subunits, A1aB1b (G1, 53.6 kd), A2B1a (G2, 52.4 kd), A1bB2 (G3, 52.2 kd), A5A4B3 (G4, 61.2 kd), and A3B4 (G5, 55.4 kd), were generated and purified as described elsewhere in detail.[E1] Briefly, the host cells BL21-(DE3), HMS174(DE3), AD494(DE3), and Origami(DE3) containing the individual expression plasmids pEA1aB1b, pEA2B1a, pEA1bB2, pEA5A4B3, and pEA3B4 were cultured in lysogeny broth or terrific broth media to an optical density of

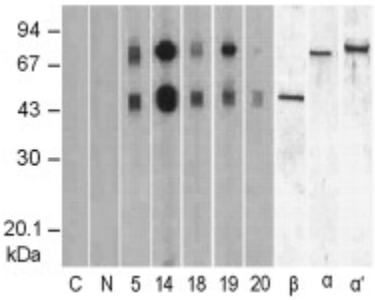
0.6 at 600 nm wavelength, and overexpression was induced by addition of 1 mmol/L isopropyl-1-thio-β-D-galactosidase. Cells were lysed by sonification and the cell debris removed by centrifugation. An initial ammonium sulfate fractionation was achieved with ammonium sulfate saturation between 15% and 60%, depending on the subunit of interest. The prefractionated recombinant proglycinins underwent hydrophobic interaction chromatography and subsequently ion exchange chromatography (A1aB1b, A2B1a, A3B4) or a combination of gel filtration and ion exchange chromatography (A1bB2, A5A4B3). The recombinant proglycinins were kept at 4°C in PBS containing 400 mmol/L NaCl until used. The recombinant proglycinin subunits of soybean glycinin G1 to G5[E1] each consisted of 1 single protein band at the anticipated molecular weight after total protein staining of the proteins (Fig E2, *A, left*). The IgE reactivity was verified with the highly sensitive serum 28 that reacted to each of the subunits Gly m 6.0101 (G1), Gly m 6.0201 (G2), Gly m 6.0301 (G3), Gly m 6.0401 (G4), and Gly m 6.0501 (G5). Also, some IgE-binding proteins of lower molecular weight in the subunits G1, G3, and G4 were apparent, which might constitute spurious amounts of degradation products because the presence of other soybean proteins in the recombinant preparations can be excluded (Fig E2, *A, right*). Immunoblot analysis of a mixture of the 5 subunits of Gly m 6 with selected patients' sera having low to moderate levels of soybean specific IgE revealed IgE reactivity around the expected molecular weight with little or no IgE reactivity to potential breakdown products (Fig E2, *B*). Assignment of the IgE reactivities to the acidic or basic chains of the subunits was not possible because each of the recombinant proglycinins was composed of 1 peptide chain with the acidic chain fused to the basic chain.

Because dissection of positive from negative results in immunoblot analyses turned out to be difficult for some sera, such as number 20 with Gly m 5 (Fig E1) and number 14 with Gly m 6 (Fig E2, *B*), an IgE-ELISA facilitating an objective cutoff proved to be more appropriate. By ELISA, the soybean major storage proteins were analyzed in a mixture representing the natural composition of these complex proteins better than the individual subunits. Gly m 5 and Gly m 6 were analyzed separately with the relevant subunits in an almost equimolar ratio. Only G2 (Gly m 6.02) was excluded from the mixture of Gly m 6 subunits because it precipitated in coating buffer. However, because of a high sequence similarity to G1 (83%) and G3 (85%), the exclusion of G2 from ELISA analysis was considered acceptable.

Reference

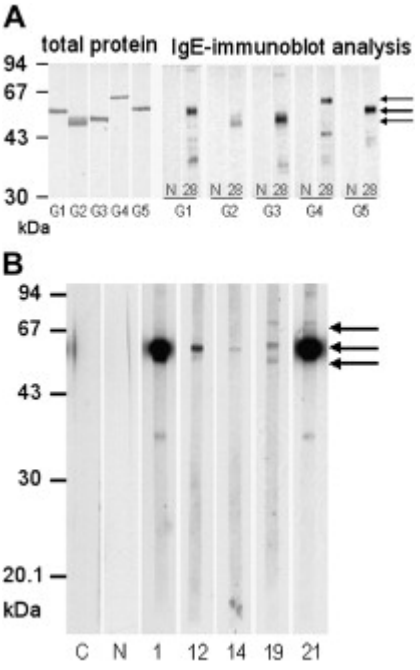
E1. Prak K, Nakatani K, Katsube-Tanaka T, Adachi M, Maruyama N, Utsumi S. Structure-function relationships of soybean proglycinins at subunit levels. J Agric Food Chem 2005;53:3650-7.

Fig E1.



Purity testing of the individual Gly m 5 (β-conglycinin) subunits β (Gly m 5.03), α (Gly m 5.01), and α′ (Gly m 5.02) from natural soybean source in SDS-PAGE and silver staining *(lanes β, α, and α')*, and verification of the IgE reactivity of all Gly m 5 subunits by immunoblot analysis with selected soy allergic sera (*lane C:* buffer control; *N:* nonatopic control serum pool [n = 3]; *lanes 5, 14, 18, 19, and 20:* subjects with soybean allergy).

Fig E2.



A, Individual subunits of Gly m 6 by total protein staining of Western blots with Ponceau S (*lanes:* G1, Gly m 6.01, G2, Gly m 6.02; G3, Gly m 6.03; G4, Gly m 6.04; G5, Gly m 6.05) and verification of IgE-reactivity of all Gly m 6 subunits *(double lane)* with patient 28 (*N:* nonatopic serum pool). **B,** IgE immunoblot analysis of equal amounts (wt/wt) of the Gly m 6 subunits with selected patients' sera (*lane C:* buffer control; *N:* nonatopic control serum pool [n = 3]; *lanes 1, 12, 14, 19, and 21:* subjects with soybean allergy). The *arrows* indicate the position of the 5 proglycinin subunits according to molecular weight.

Table E1.

Identified IgE-reactive clones from the soybean cDNA expression library

	Glycinin		**β-Conglycinin**		
	A1aBx	**A2B1a**	**α Subunit**	**α Prime**	**β Subunit**
Alignment hit	**G1 (precursor)**	**G2 (precursor)**	**(precursor)**	**subunit (precursor)**	**(precursor)**
GenBank acc. no.	X02985	D00216	AB008678	AB008680	AB008679
No. of primary IgE-reactive clones	1	2	5	6	9
Average size of insert (bp)	426	736	515	304	473

Full-size table

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Disclosure of potential conflict of interest: C. Bindslev-Jensen has served as an expert witness in a patient antihistamine injury case. L. K. Poulsen has served as an

expert witness for Novozymes Ltd. S. Vieths is an associate at the Institute for Product Quality Berlin; has received honoraria from Phadia and the Food Allergy Resource and Research Program; has received research support from the European Union, the Germany Research Society, the Research Fund of the German Food Industry, Monsanto Co, and the European Directorate for the Quality of Medicine and Health Care; and has served as a member of the European Academy of Allergy and Clinical Immunology, the International Union of Immunological Diseases, the European Agency for the Evaluation of Medicinal Products, the European Pharmacopoeia Commission, the International Life Sciences Institute Health and Environmental Science Institute, the European Committee for Standardization, and Deutsche Gesellschaft fur Allergie und Klinische Immunologie. The rest of the authors have declared that they have no conflict of interest.